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(54) Title: ISOTHERMAL TRANSCRIPTION BASED ASSAY FOR THE DETECTION AND QUANTIFICATION OF CHEMOKINE RANTES, MIP-1 α AND MIP-1 β		
(57) Abstract An isothermal transcription based amplification assay for the detection or quantification of chemokine RNA uses primers and probes for sequences within the gene for RANTES, MIP-1 α and MIP-1 β . The quantitative system uses an internal control Q of a mutant version of each gene. Target specific primers and probes are also disclosed.		

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ISOTHERMAL TRANSCRIPTION BASED ASSAY FOR THE DETECTION
AND QUANTIFICATION OF CHEMOKINES RANTES, MIP-1 α and
MIP-1 β

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FIELD OF THE INVENTION

The present invention is directed to an isothermal transcription based assay for the detection and quantitation of the β chemokines regulated-upon-activation, normal T expressed and secreted (RANTES), macrophage inflammatory protein-1 α (MIP-1 α) and macrophage inflammatory protein-1 β (MIP-1 β). The present invention is also directed to oligonucleotides for amplifying chemokine RNA and probes for use in the detection and quantitation of the amplification product.

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BACKGROUND OF THE INVENTION

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The RANTES chemokine has been shown to be a potent inhibitor of HIV-1 infection (Cocchi et al, *Science* 270:1811, 1996). In this study it was shown that RANTES is one of the major suppressor factors produced by CD8+ cells. These suppressor factors have been implicated in the long term nonprogression of many asymptomatic HIV-1 + patients.

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The invention described herein is a transcription based amplification method for the detection of the RANTES mRNA transcript, which will be detected in the PBL of HIV-1 + patients. The purpose of applying such an assay would be to determine the presence and level of the transcripts and use these results to predict prognosis and/or direct therapeutic management of the infected patient. The invention would involve primers that were found to be optimal for the detection/quantification of the RANTES transcript in vivo using NASBA. Qualitative and quantitative detection would involve the use of an internal control RNA standard. The preliminary sample processing and nucleic acid isolation would be as described by Boom et al. The

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detection technology would involve the colorimetric ELGA based system and the ECL based system.

5 The β chemokines MIP-1 α , MIP-1 β and RANTES inhibit infection of CD4⁺ T cells by primary, non-syncytium-inducing HIV-1 strains at the virus entry stage, and also block env-mediated cell-cell membrane fusion. CD4⁺ T cells from some HIV-1-exposed uninfected individuals cannot fuse with NSI HIV-1 strains and secrete high levels of β -chemokines. Expression of the β -chemokine receptor CC-CKR-5 in
10 CD4⁺, non-permissive human and non-human cells renders them susceptible to infection by NSI strains, and allows env-mediated membrane fusion. CC-CKR-5 is a co-receptor for NSI primary strains of HIV-1. (Dragic et al, *Nature*, 381:667 (1996)).

15 The super family of chemoattractant cytokines (chemokines) and their receptors are involved in inflammation and infection. The chemokines range in size from 68 to 120 amino acids (in the mature form) and can be divided into three classes based on variations in a shared cysteine motif. The largest group, the C-C, or β chemokines has nearly 20 members identified to date. The smallest group, the C class,
20 has but one. The C-X-C, or α chemokine branch can be further subdivided into two groups based on structure and function. The largest of these groups contains proteins containing the E-L-R-C-X-C motif and the smaller group is made up of proteins without the E-L-R amino terminal to C-X-C. The structural classes parallel function to a
25 large extent in that most C-X-C chemokines with E-L-R are chemoattractants for neutrophils but not monocytes, whereas C-C chemokines generally attract monocytes and lymphocytes, but not neutrophils. Basophils and eosinophils are also affected predominantly by C-C chemokines. The C chemokine appears thus far to be
30 lymphocyte specific.

5 A number of chemokine receptors have also been identified. These proteins are structurally related, with amino acid homology high in the transmembrane regions and some intracellular loops. There seems to be less homology at the N- and C-termini, and extracellular loops, which are presumed to be involved in ligand binding (N-terminal) and receptor specific interactions with signaling components.

10 Engagement of the chemokine receptors results ultimately in the movement of the cell. The steps of this process are thought to be: relay of information from the receptor through G-proteins; mobilization of intracellular second messengers; coordinated reorganization of the cytoskeleton; formation of focal adhesions and attachment to and detachment from the substrate with pseudopodial extension, and retraction to effect directional migration.

15 The involvement of the chemokines and chemokine receptors in HIV infection is beginning to be understood. It has been known for some time that binding of the viral glycoprotein gp120 to CD4 is not sufficient for viral fusion and entry, suggesting the need for an additional coreceptor for HIV infection. Isolates of HIV generally have a tropism for either transformed T-cells (T cell line tropic or simply T-tropic strains) or for monocytes or cultured macrophages, and primary T cells, but not transformed T cells (macrophage tropic). The difference in tropism appears to reside in the V3 region of the gp120.

25 It was also known that there were endogenous HIV suppressor factors secreted by CD8⁺ T cells that inhibited viral replication in CD4⁺ cells *in vitro*. The β chemokines RANTES, MIP-1 α and MIP-1 β have now been identified as soluble suppressors of macrophage-tropic, but not T cell line tropic, HIV infection *in vitro*. (reviewed in Premack, *Nature Medicine*, 2(11):1174 (1996)).

30 It has been observed that the resistance of persons who remain uninfected despite multiple high-risk sexual exposures is associated with the activity of the C-C chemokines RANTES, MIP-1 α and MIP-1 β . The

relative resistance does not extend to T-cell line-adapted strains (Paxton et al., *Nature Medicine*, 2(4): 412 (1996)). These results are consistent with a model in which CCR5 is a coreceptor (with CD4⁺) for macrophage tropic strains of HIV, and RANTES, MIP-1 α and MIP-1 β are also ligands to CCR5. It has been shown that RANTES, MIP-1 α and MIP-1 β block the entry of HIV-1 into cells (Cocchi et al, *Nature Medicine*, 2(11):1244 (1996)).

Chemokine levels in infected patients are therefore very important in determining the course of the disease. Detecting and quantitating the RNA for the chemokines is therefore an important factor in the clinical evaluation of HIV positive patients.

Because chemokines are involved in the activation of inflammatory cells, previous workers have investigated the levels of chemokines or chemokine RNAs in different diseases or conditions related to inflammation. These diseases and conditions include graft rejection (Strehlau et al, *Proc. Nat. Acad. Sci.* 94(2): 695-700 (1997)); cancer (Burket et al, *Cytokine* 8(7): 578-585, (1996)); HIV-1 infection (Schmidtayerova et al, *Proc. Nat. Acad. Sci.* 93(2):700-704, (1996)); human cytomegalovirus infection (Michelson et al, *J. Virol.* 71(9):6495-6500 (1997)); asthma (Berkman et al, *Am. J. Respir. Crit. Care Med.* 154(6/1):1804-11 (1996); and others.

Lastly, it may be easier to establish correlations between β -chemokines transcript levels and disease state due to the increased sensitivity of the amplification based transcript NASBA assay, and the typically larger dynamic range of the quantitative NASBA system.

An isothermal amplification method for detection or quantification of chemokine mRNAs has not been described.

SUMMARY OF THE INVENTION

The present invention provides isothermal transcription based amplification assays for the detection and quantitation of β chemokine

5 RNAs. The detection assay uses primer pairs and probes for each of the targets, RANTES, MIP-1 α and MIP-1 β . Quantitative assays use an internal control RNA (Q) that differs from the wild type RNA by a small internal randomized segment. Q RNA is spiked into the sample at a known copy number at lysis and is coextracted and coamplified with wild type RNA. The resulting amplicate is then subjected to two independent hybridization reactions with probes specific for the wild type and Q amplicates. Quantitation of the wt RNA is achieved by calculating the ratio of resulting signal of wild type to Q.

10 An isothermal amplification method starts with an RNA template and alternately synthesizes DNA and RNA. Using an RNA template, a primer, and reverse transcriptase, an RNA/DNA hybrid is generated. The RNA is degraded from the hybrid by the RNase H activity. A double stranded DNA is then generated by the reverse transcriptase using another primer, and then the double stranded DNA is used as template for large amounts of RNA synthesis by the RNA polymerase. One of the primers has, in addition to the sequences complementary to the template, additional sequences necessary for generating an RNA polymerase promoter and transcription initiation site which can be used by the RNA polymerase.

15 One of the advantages of an isothermal transcription based amplification method, as compared to other amplification methods such as PCR, is that by being essentially isothermal, it requires few manipulations by the experimenter. The method may be used on purified or semi-purified RNA extracts, or on cell or tissue samples with *in situ* amplification. In addition, if the sample contains both DNA and RNA, the use of RT/PCR requires a first step of DNase treatment, or some method to distinguish the amplification products of mRNA- and DNA-derived PCR products is necessary. DNase treatment prior to RT-PCR can be employed (Bitsch, A. et al., *J. Infect. Dis.* 167, 740-743, 1993;

Meyer, T. et al., *Mol. Cell Probes*, 8, 261-271, 1994), but sometimes fails to remove contaminating DNA sufficiently (Bitsch, A. et al., 1993).

The purpose of applying such an assay would be to determine the presence and level of these transcripts, and to use these levels as prognostic makers (in conjunction with other disease makers) and/or to direct therapeutic management of the infected patient.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows the specificity of the NASBA chemokine RNA assays discussed in Example 5.

FIGURE 2 shows the quantitation of RANTES *in vitro* transcribed RNA discussed in Example 2A.

FIGURE 3 shows the results of the RANTES NASBA assay on PBMCs from 12 HIV+ patients discussed in Example 2B.

FIGURE 4 shows the quantitation of MIP-1 α *in vitro* transcribed RNA discussed in Example 3B.

FIGURE 5 shows the results of the MIP-1 α NASBA assay on PBMCs from 12 HIV+ patients discussed in Example 3C.

FIGURE 6 shows the quantitation of MIP-1 β *in vitro* transcribed RNA discussed in Example 4B.

FIGURE 7 shows the results of the MIP-1 β NASBA assay on PBMCs from 3 HIV+ patients discussed in Example 4C.

FIGURE 8 compares the RANTES and MIP-1 α RNA levels in HIV-1+ patients.

DETAILED DESCRIPTION OF THE INVENTION

An isothermal transcription based assay is used for the detection and genotyping of dengue RNA. Any isothermal transcription based assay may be used with the primers and probes of the present invention.

The isothermal transcription based assay of the present invention is

carried out under conditions that can be readily determined by a person of ordinary skill in the art.

5 The preferred amplification method of the present invention is the isothermal transcription based amplification system referred to as NASBA. The NASBA method is disclosed in U.S. Patent Nos. 5,409,818 and 5,554,527, which are herein incorporated by reference. NASBA includes the use of T7 RNA polymerase to transcribe multiple copies of RNA from a template including a T7 promoter.

10 Another technique for the amplification of nucleic acid is the so-called transcription based amplification system (TAS). The TAS method is described in International Patent Application No. WO 88/10315. Transcription based amplification techniques usually comprise treating target nucleic acid with two oligonucleotides one of which comprises a promoter sequence, to generate a template including a functional promoter. Multiple copies of RNA are transcribed from said template and can serve as a basis for further amplification.

15 Other transcription based amplification techniques are described in EP 408295. EP 408295 is primarily concerned with a two-enzyme transcription based amplification method. Transcription based amplification methods, such as the NASBA method described in EP 329822, are usually employed with a set of oligonucleotides, one of which is provided with a promoter sequence that is recognized by an enzyme with DNA dependent RNA polymerase activity such as, for example, T7 polymerase. Several modifications of transcription based techniques are known in the art. These modifications comprise, for example, the use of blocked oligonucleotides (that may be provided with a promoter sequence). These oligos are blocked so as to inhibit an extension reaction proceeding therefrom (US 5,554,516). One or more "promoter-primers" (oligonucleotides provided with a promoter sequence) may be used in transcription based amplification techniques,

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optionally combined with the use of one or more oligonucleotides that are not provided with a promoter sequence.

The term "oligonucleotide" as used herein refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides.

5 Such oligonucleotides may be used as primers and probes.

Of course, based on the sequences of the oligonucleotides of the present invention, analogues of oligonucleotides can also be prepared. Such analogues may constitute alternative structures such as "PNA" (molecules with a peptide-like backbone instead of the phosphate sugar backbone of normal nucleic acid) or the like. It is evident that these alternative structures, representing the sequences of the present invention are likewise part of the present invention.

15 The term "primer" as used herein refers to an oligonucleotide either naturally occurring (e.g., as a restriction fragment) or produced synthetically, which is capable of acting as a point of initiation of synthesis of a primer extension product which is complementary to a nucleic acid strand (template or target sequence) when placed under suitable conditions (e.g., buffer, salt, temperature and pH) in the presence of nucleotides and an agent for nucleic acid polymerization, such as DNA dependent or RNA dependent polymerase. A primer must be sufficiently long to prime the synthesis of extension products in the presence of an agent for polymerization. A typical primer contains at least 10 nucleotides in length of a sequence substantially complementary or homologous to the target sequence, but somewhat longer primers are preferred. Usually primers contain about 15-26 nucleotides but longer primers may also be employed, especially when the primers contain additional sequences such as a promoter sequence for a particular polymerase.

30 Normally a set of primers will consist of at least two primers, one "upstream" (P2) and one "downstream" (P1) primer which together define the amplificate (the sequence that will be amplified using said

primers). One of the primers is understood to contain, in addition to sequences that will hybridize to the target sequence, sequences which provide promoter activity. Most often the P1 primer will include the promoter sequence.

5 The term "promoter sequence" defines a region of a nucleic acid sequence that is specifically recognized by an RNA polymerase that binds to a recognized sequence and initiates the process of transcription by which an RNA transcript is produced. In principle, any promoter sequence may be employed for which there is a known and available
10 polymerase that is capable of recognizing the initiation sequence. Known and useful promoters are those that are recognized by certain bacteriophage RNA polymerases such as bacteriophage T3, T7 or SP6. Their function as a primer, e.g., the starting point for an elongation reaction, however, may be blocked, as already mentioned above, or
15 absent in some embodiments of transcription based amplification reactions. A particularly preferred promoter sequence is the sequence of the T7 RNA polymerase promoter, the sequence of which is as follows: *AATTCTAATACGACTCACTATAGGG* SEQ ID NO:44.

20 SEQ ID NOs 4, 10, 16, 18, 22, 27, 33, 35, and 42 comprise the sequences of the specific target primer operably linked to the T7 promoter sequence, shown in italics. This makes the sequences especially suitable for use as downstream primer in a transcription based amplification technique such as NASBA. However, the sequences for
25 the T7 promoter shown in italics may be substituted by sequences of other promoters for use with other RNA polymerases.

A preferred embodiment of the present invention is a combination of two oligonucleotides according to the invention, for use as a set in nucleic acid amplification.

30 One of the oligonucleotides may serve as an "upstream oligonucleotide", i.e., upstream primer, while the second oligonucleotide

serves as a "downstream oligonucleotide", i.e., downstream primer, in the amplification reaction.

5 Preferably, the reverse transcriptase activity is provided by avian myeloblastosis virus (AMV) reverse transcriptase and the RNA polymerase is provided by T7 RNA polymerase.

One of the advantages of an isothermal transcription based amplification method, as compared to other amplification methods such as PCR, is that by being isothermal, it requires few manipulations by the experimenter. However, the absence of a high temperature step does
10 make it somewhat more difficult to find appropriate primers (see below).

The amplification method of the present invention may be applied to extracts of samples comprising nucleic acid, or whole cells or tissues for *in situ* amplification. The samples may be various body fluids,
15 particularly blood, plasma, and serum, from humans. The samples may also be tissue samples from humans, for instance, lymph tissue.

If the method is applied to extracts of samples comprising nucleic acids, the sample may be total RNA extracts (such as those described in Chomczynski and Sacchi, *Anal. Biochem.* **162**:156, 1987) or "Boom"
20 extracts (Boom et al, *J. Clin. Micro.*: **28**, No.3, March 1990, p.495-503), which is herein incorporated by reference. The method is preferably applied to "Boom extracts".

The amplificate is detected by hybridization with an appropriately labeled oligonucleotide probe. The label may contain a radioactive moiety, a detectable enzyme, or any other moiety capable of generating
25 a detectable signal, such as a colorimetric, fluorescent, chemiluminescent or electrochemiluminescent (ECL) signal. Blot based hybridization analysis and liquid hybridization based ECL analysis are preferably used, although other analysis systems such as ELGA
30 (enzyme-linked gel assay) and *in situ* hybridization can also be used.

In one embodiment of the present invention, the amplification products are resolved by agarose gel electrophoresis, then transferred to nylon membranes and hybridized to a probe that is 5'-end labeled with ^{32}P using standard methods. The products are then visualized by autoradiography. In a second embodiment of the present invention, the amplification products can be detected using the ELGA. In this method the products of the amplification reaction are resolved on a polyacrylamide gel. A probe that is specific for the amplification reaction product and conjugated at its 5' end with horseradish peroxidase (HRP) is then hybridized. A colorimetric enzyme reaction allows for the visualization of the reaction product in the gel. A third embodiment of the present invention makes use of electrochemiluminescence chemistry (or ECL). This embodiment uses a biotinylated capture probe immobilized onto the surface of a streptavidin-coated magnetic bead via the biotin-avidin interaction. This system also requires an oligonucleotide detector probe, which can hybridize to an independent region of the amplification product. This detector probe is labeled with Ruthenium, the substance that is responsible for generating an ECL signal. Each of the detection systems uses one or more internal controls to monitor the efficiency of the extraction process and the amplification assay itself. The detection systems are described in detail in Romano et al, DNA Technology 16:89-103 (1996), and van Gemen et al., J. of Virol. Methods, 49:157-168 (1994), which are herein incorporated by reference. Methods for internal controls are described in van Gemen et al, Reviews in Medical Virology, 5:205-211 (1995), which is herein incorporated by reference.

It may also be relevant to adapt the assay for an *in situ* format, which would be useful in pathology studies of tissue, particularly for lymphatic tissues. If the method is to be practiced on fixed preparations for *in situ* analysis, the method is performed as follows. Samples may include various body fluids or tissue samples. Lymph tissue is a preferred

tissue for *in situ* analysis. The cells are fixed and then permeabilized to optimize permeability of the cell membranes. The fixatives are those standardly used in the art for cell or tissue preparations, such as acetone and methanol, ethanol, formalin, formaldehyde, paraformaldehyde, or Permafix®, and the permeabilization is done by proteinases, such as proteinase K or pepsinogen. The cells are then washed to remove all reagents that might inhibit the transcription based reaction. Permeabilization is done to the point that the cells allow entry of all necessary amplification reaction components, yet retain the targets and amplification products within the cells. In addition, cosolvents such as glycerol or DMSO may be added to optimize the NASBA reaction.

Detection of amplification products may be by direct labelling (with, for instance, biotin or digoxigenin - UTP) or by *in situ* hybridization with labelled probe. The direct labelling method requires that conditions can be optimized to remove unincorporated label while maintaining the amplification products.

In a particularly preferred embodiment of the present invention, the isothermal transcription based amplification method is used in concert with a particular RNA extraction technique ("Boom extraction", Boom et al, *J. Clin. Micro.*: 28, No.3, March 1990, p.495-503), and ECL detection (electrochemiluminescence). The advantages of the system are those associated with an amplification based assay capable of providing sequence level data. Although some of these same advantages exist for the RT-PCR (i.e., increased sensitivity over ELISA, gene sequence specificity), there are advantages of NASBA for RNA over RT-PCR. These include isothermal amplification, incorporation of reverse transcription into the amplification, application to wider array of specimen types (via Boom extract), and the sensitivity and dynamic range of the ECL detection.

Boom extracts are purified preparations of DNA and RNA. The Boom method is based on the lysing and nuclease inactivating properties

of the chaotropic agent guanidinium thiocyanate (GuSCN) together with the nucleic acid binding properties of silica particles or diatoms. By using size fractionated silica particles, nucleic acids, including covalently closed circular, relaxed circular, linear double-stranded DNA, single stranded DNA, tRNA, mRNA, and rRNA, can be purified from a sample
5 in less than one hour and recovered in the original reaction vessel.

A small sample is pipetted into a reaction vessel containing a solid nucleic acid carrier and a GuSCN containing lysis buffer. Lysis of the cells occurs and the released nucleic acids bind to the carrier. The
10 carrier-nucleic acid complexes can be separated by centrifugation. Several wash steps follow and the complexes are then dried. The nucleic acids are eluted in an aqueous low-salt buffer in the initial reaction vessel and used for the amplification reaction.

In a preferred embodiment of the present invention, amplification
15 is achieved in a 20 μ L reaction containing 5 μ L of the nucleic acid extract material in 10 μ L of premix [Tris (40 mM) pH8.5; $MgCl_2$ (12 mM); KCl (70 mM); DTT (5mM); dNTPs (each) (1 mM); rATP, rUTP, rCTP (2 mM); rGTP (1.5 mM); ITP (0.5 mM); DMSO (15%); P1 and P2, (0.2 μ M); Sorbitol (1.5 M)]. This is then added to 5 μ L of enzyme mix
20 [BSA (2.1 μ g/NASBA); RNase H (0.08 unit/NASBA); T7 RNA Polymerase (32 units/NASBA); and AMV-RT (6.4 units/NASBA)]. (The enzyme mixture must not be vortexed). If the nucleic acid sample decreases (5 μ l), then the water volume increases accordingly so that the total volume stays 15 μ l when the nucleic acid is added.

25 The method can be carried out as follows.

1. Mix premix.
2. Add 10 μ l of premix to 5 μ l of nucleic acid in an Eppendorf tube.
3. Incubate at 65 °C for 5 minutes.
4. Transfer to 41 °C heat block, incubate for 5 minutes.
- 30 5. Add 5 μ l of enzyme mix.
6. Mix without vortexing.

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7. Incubate at 41 °C for 5 minutes.
8. If the tops of the tubes have condensation from the cooling, they may be spun.
9. Incubate at 41 °C for 90 minutes.
- 5 10. Spin down samples and store at -20°C.

In the method of the present invention primers and probes were designed for the transcripts of RANTES, MIP-1 α and MIP-1 β . The primer and probe sequences were derived from the Genbank entries for these genes. A total of six primers were initially designed and synthesized; there was one primer combination for each of the three different target sequences. Subsequently additional primers and probes were also made. The primers and probes are listed on Table 1.

15 Table 1: Oligonucleotides

<u>RANTES</u>			
20	Oligo	(Map Site*)	Sequence (all listed 5' to 3')
	P2A	(88-112)	ACCACACCCTGCTGCTTTGCCTACA [SEQ ID NO:1]
	P1A	(296-320)	AAACAGGCAAATTTGTGTAAGTTCA [SEQ ID NO:2]
25	WT PROBE	(194-213)	TCACCCGAAAGAACCGCCAA [SEQ ID NO:3]
	P1A*		AATTCTAATACGACTCACTATAGGGAAACAGGCAAATTTGTGTAAG
30	TTCA		[SEQ ID NO:4]
	P2B	(125-146)	TGC CCC GTG CCC ACA TCA AGG A
			[SEQ ID NO:5]
35	WT PROBE	(194-213)	TCA CCC GAA AGA ACC GCC AA
			[SEQ ID NO:6]
	Q PROBE		TCA CAC AGG CAC CGA ACC AA [SEQ ID NO:7]

15

CAPTURE PROBE (225-249) CCC AGA GAA GAA ATG GGT TCG
GGA G [SEQ ID NO:8]

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MIP-1 α

Oligo (Map Site*) Sequence (all listed 5' to 3')

10 P1A (273-296) AGAGCGAAGCTTCTGGACCCCTCAGGCA
[SEQ ID NO:9]

P1A*

15 AATTCTAATACGACTCACTATAGGGAGAGCGAAGCTTCTGGACCCC
TCAGGCA [SEQ ID NO:10]

P2A (138-167) CATAGCTGACTACTTTGAGACGAGCAGCCA
[SEQ ID NO:11]

20

PROBE (232-256) GAGGAGTGGGTCCAGAAATATGTCA
[SEQ ID NO:12]

25 P2C (349-372) GGG AAC ATG CGT GTG ACC TCC ACA 3'
[SEQ ID NO:13]

P2D (397-420) TTG CCA AAC AGC CAC ACT GTG GGA 3'
[SEQ ID NO:14]

30 P1C (500-524) ACA GGG GAA CTC TCA GAG CAA ACA A3'
[SEQ ID NO:15]

P1C* AATTCTAATACGACTCACTATAGGGACAGGGGAAGTCTCA
GAGCAAACA A [SEQ ID NO:16]

35

P1D (561-585) ACA CAG GCT GAT GAC AGC CAC TCG G
[SEQ ID NO:17]

40 P1D* AATTCTAATACGACTCACTATAGGGACACAGGCTGATGACAGCCAC
TCG G [SEQ ID NO:18]

PROBES

45 WT probe 2 (475-496) TTT CGA TTT CAC AGT GTG TTT G[SEQ
ID NO:19]

Q probe TTT CGT CAT TTA TAG CGG TTT G [SEQ ID NO:20]

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Oligo (Map Site*)	Sequence (all listed 5' to 3')
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TCG CGG TGT A [SEQ ID NO:33]

P1C (196-220) GCT GGC TGG GAG CAG AGG CTG CTG G
[SEQ ID NO:34]

5

P1C* AATTCTAATACGACTCACTATAGGGGCT GGC TGG GAG
CAG AGG CTG CTG G [SEQ ID NO:35]

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P2E (-15-6) TTC TGA GTT CTG CAG CCT CAC C
[SEQ ID NO:45]PROBES

15

WT probe 2 (71-95) GCT AGT AGC TGC CTT CTG CTC TCC A
[SEQ ID NO:36]WT probe 2B (72-93) CTA GTA GCT GCC TTC TGC TCT C
[SEQ ID NO:37]

20

Q probe CTA GTT CGA TTC GGT CCC TCT C
[SEQ ID NO:38]WT probe (72-93) CTA GTA GCT GCC TTC TGC TCT C
[SEQ ID NO:39]

25

Capture probe (44-63) CTG CGT GAC TGT CCT GTC TC
[SEQ ID NO:40]

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P1B (347-370) GGAGAAGCATCCGGGCTCAGGTGA
[SEQ ID NO:41]P1B*
AATTCTAATACGACTCACTATAGGGGGAGAAGCATCCGGGCTCAGG
TGA [SEQ ID NO:42]

35

P2B (185-214) TTACTATGAGACCAGCAGCCTCTGCTCCCA
[SEQ ID NO:43]

40

*Position 1 is the A in the initiator codon.

Example 1 - NASBA-Initial Evaluation

A. Detection of Chemokine Transcripts By NASBA

Primers for RANTES (P1A/P2B for all cells except the B cells, which used P1A/P2A) and MIP-1 α (P1A/P2A) were made and used in the standard NASBA assay on RNA prepared by the method of Chomczynski and Sacchi (1987) or by the method of Boom et al. (1990). Samples tested were HTLV-I chronically infected T-cells (MT2), HTLV-II chronically infected T-cells (MoT), HIV-1 chronically infected H9 cells (H9+), H9 cells, three patient PBMC sample (P1, P2, P3), PHA stimulated Peripheral Blood Mononuclear Cells (PBMC), enriched CD4+, CD8+, and B-cell populations, SF9 (negative control insect cells), human colon adenocarcinoma (COLO-205), human prostate adenocarcinoma (PC-3), and metastatic prostate adenocarcinoma (LNCAP). As shown on Table 2, almost all cell lines (other than the negative control, SF9) tested positive for both RANTES and MIP-1 α .

Table 2: Detection of Chemokine
Transcripts by NASBA

Source	RANTES	MIP-1 alpha	Source	RANTES	MIP-1 alpha
MoT	pos	pos	PBMC	pos	pos
MT2	pos	pos	CD4+	pos	pos
H9+	pos	pos	CD8+	pos	pos
H9	pos	neg	B cells	pos	pos
Colo 205	pos	neg	Patient 1	pos	pos
PC 3	pos	pos	Patient 2	pos	pos
LNCAP	pos	pos	Patient 3	pos	pos
SF9	neg	neg			

B. Specificity of the MIP-1 α and RANTES NASBA Assays

Wild type, *in vitro* produced RNA was obtained from plasmid constructs encoding the MIP-1 α , RANTES, and MIP-1 β cDNAs. Approximately 10⁵ copies of each of these RNAs were then amplified by NASBA with either the MIP-1 α or RANTES primers, as above. Reaction products were then subjected to hybridization detection analysis using the ECL system, as well as ³²P labeled detector probes in Northern analysis. Each primer set was specific for the appropriate target RNA (data not shown).

10 C. RANTES WT and Q RNA NASBA Specificity

As shown in part A above, since it appeared that RANTES transcript was virtually ubiquitous, a quantitative assay was desired. Following the strategy outlined earlier, RANTES cDNA (WT) was cloned and mutagenized so as to produce the Q RNA calibrator. RNA was produced *in vitro* from both of these plasmid constructs. Further, ECL detection systems specific for RANTES WT and Q RNAs were developed. To evaluate this ECL system, 10⁶ copies of WT and Q RANTES RNAs were amplified by NASBA using the P1A/P2B primer pair. Resulting amplicates were analyzed by the WT and Q ECL detection system. Results show these probes are specific for WT and Q amplicates (data not shown).

20 D. RANTES WT and Q NASBA Sensitivity

The lower limit of amplification with the RANTES NASBA primer set was determined separately for *in vitro* RANTES WT and Q RNAs. Ten-fold dilutions of OD₂₆₀ quantified RNA (WT & Q) were amplified in separate NASBA reactions and analyzed with the ECL detection system. After correction for ECL background signal, it was demonstrated that amplification of both WT and Q RANTES RNAs was possible down to approximately 50 input copies (data not shown).

E. NASBA Coamplification of RANTES WT and Q RNA

In order to utilize an internal Q RNA standard for quantitative NASBA, it must be demonstrated that the amplification and detection efficiencies of the standard are sufficiently similar to the RNA analyte of interest. To this end, ten-fold dilutions of *in vitro* produced WT RANTES RNA were coamplified with either 2.5×10^5 or 2.5×10^4 copies of Q RNA. After coamplification, the reaction products were analyzed separately by ECL with WT and Q specific probes. The resulting ECL ratios were then used to calculate the WT copy number. Results demonstrated that the calculated copy number for the WT RNA was consistent in the undiluted sample. Further, the assay demonstrated ten-fold changes in RNA level, consistent with the dilution scheme used (data not shown).

F. Quantitation of Control RANTES RNA Subjected to Boom Extraction

Since actual clinical samples require a nucleic acid isolation step, the RANTES QT NASBA assay must be functional on material processed by the Boom method. This was demonstrated by essentially repeating the model system experiment described in section D, however the RNA mixtures were first subjected to coextraction, followed by coamplification. By establishing the ratio of WT RNA to the known Q RNA spike at the time of processing in lysis buffer, the assay is normalized for the potential loss of material during the course of the extraction procedure. The ECL ratios were used to calculate the WT RNA copy.

G. RANTES QT NASBA on PBMC and CEM

Once quantitation was shown to work satisfactorily with *in vitro* RNA model systems, PBMC and CEM (human carcinoma embryo) cells were analyzed. Ten-fold dilutions were made of each cell source in pH

6.2 GuSCN lysis buffer. A 2.5×10^5 Q spike was added to each dilution sample and subjected to coextraction by the Boom method. ECL signals for PBMC and CEM were used to quantitate WT RNA. Importantly, an approximate ten-fold change in WT RNA level was found, which is consistent with the ten-fold dilution scheme (data not shown).

Example 2 RANTES

After looking at sequence comparisons with the MIPs and RANTES, P2B was designed to be less homologous to the MIP sequences. Sensitivity was tested and found to be the same as P2A, that is, 5 pg F3B (HTLV-1 transformed CD8+ cell line derived from an HIV-1 positive patient) total RNA. P1A/P2B was selected as the preferred primer set and all subsequent work was done with this set.

A. The primer oligonucleotides P1A and P2B were used with the Q control to quantitate RANTES *in vitro* produced RNA. *In vitro* transcribed RNA from the wild type gene as well as the Q gene was co-amplified with three different concentrations of the Q RNA. After amplification each mixture was measured by ECL with the correct probe.

The results are shown on Figure 2. The mean log measured is proportional to the mean log input of RNA.

B. The same quantitative system was then used to measure RANTES RNA in dilutions of patient PBMCs. The PBMCs from twelve patients that were HIV-1+ as assessed by clinical diagnosis were used in a NASBA assay. RNA was extracted by the Boom method from the PBMCs and amplifications were performed on RNA from 10^4 cells, 10^3 cells, and 10^2 cells for each patient. The log mean value was then derived from individual analysis of 10^4 , 10^3 , and 10^2 PBMCs per patient

and results normalized to 10^4 PBMCs. These results are shown in Figure 3.

5 C. Interestingly, the RANTES primer set will also amplify the simian version of this gene, suggesting that the invention may also be useful in monitoring suppressor factor production in animal model systems (data not shown).

Example 3 MIP-1 α

10 A. The sensitivity of the P1A/P2A primer pair was found to be 5 pg F3B total RNA. However, the primer pair did not demonstrate good *in vitro* RNA sensitivity. Additional primer pairs were designed and made. P1B/P2B were designed around the same area of the RNA as the P1A/P2A primer pair. Sensitivity was again found to be very poor. Additional primer pairs were designed and made in a separate region of the RNA. These latter primer pairs, P1C/P2C and P1D/P2D, showed good sensitivity, several orders of magnitude better than the prior pairs. P1C/P2C was selected for future use because there was more room for a capture probe with this pair.

20 B. The primer oligonucleotides P1C and P2C were used with the wt probe 2 and the Q control to quantitate MIP-1 α *in vitro* produced RNA. *In vitro* transcribed RNA from the wild type gene as well as the Q gene was co-amplified with three different concentrations of the Q RNA. After amplification each mixture was measured by ECL with the correct probe. The results are shown on Figure 4. The mean log measured is proportional to the mean log input of RNA.

25 C. The same quantitative system was then used to measure MIP-1 α RNA in dilutions of patient PBMCs. The PBMCs from twelve patients that were HIV-1 + as assessed by clinical diagnosis were used in a NASBA assay. RNA was extracted by the Boom method from the

30

PBMCs and amplifications were performed on RNA from 10^6 cells, 10^5 cells, and 10^4 cells for each patient. The log mean value was then derived from individual analysis of 10^6 , 10^5 , and 10^4 PBMCs per patient and results normalized to 10^6 PBMCs. These results are shown in Figure 5.

Example 4 MIP-1 β

A. The first primer pair designed and made, P1A/P2A, was found to be of poor specificity. Even though the sequence did not predict such problems, the primer set amplified RANTES, MIP-1 α and MIP-1 β detected with the β probe.

A second set of primers was designed, made and tested. These primers, P1B/P2B, showed good specificity and acceptable sensitivity for *in vitro* transcribed RNA, but the primer set could not amplify cellular samples in NASBA.

Primers P1D, P2D, P2C were designed from a new area of the RNA. Both primer sets showed the same sensitivity. P1D/P2C was selected for additional work because the P2D could then be used as capture probe.

B. The primer oligonucleotides P1D and P2C were used with the Q control to quantitate MIP-1 β *in vitro* produced RNA. *In vitro* transcribed RNA from the wild type gene as well as the Q gene was co-amplified with three different concentrations of the Q RNA. After amplification each mixture was measured by ECL with the correct probe.

The results are shown on Figure 6. The mean log measured is proportional to the mean log input of RNA.

C. The same quantitative system was then used to measure MIP-1 β RNA in dilutions of patient PBMCs. The PBMCs from three patients that were HIV-1 + as assessed by clinical diagnosis were used in a NASBA

assay. RNA was extracted by the Boom method from the PBMCs and amplifications were performed on RNA from 10^6 cells, 10^5 cells, and 10^4 cells for each patient. These results are shown in Figure 7.

5 Example 5. Specificity of the Preferred Primer Pair

The preferred primer pairs of Examples 2, 3, and 4 above were used to test the specificity of the NASBA assay of In vitro transcribed RNA. As shown in Figure 1, each of the systems is specific for its target RNA.

10

The biggest problem encountered in the development of NASBA assays is the selection of primers. It has often been the case that primers selected from sequence data, and meeting all the known requirements for primers, do not actually function in practice (such as was the case with MIP-1 α primers noted above). In addition, in some cases primers have been developed using model systems such as *in vitro* transcribed RNA, virus stocks, or cells lines with very high expression of the target gene, but those primers were found to be nonfunctional when the target molecule is in a background of clinical samples (such as was the case with the MIP-1 β primers noted above). The exact mechanism underlying this problem is not understood, but is believed to arise due to the lower temperature of the NASBA reaction, which does not entirely melt secondary structure of the target molecule and/or allows nonspecific binding of primers to background nucleic acids in the sample. It is essential for the application of the NASBA system to clinical samples that the primers be not absorbed by background nucleic acids, but rather be available for specific binding to the target molecule.

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The results shown in the present application demonstrate that the primers and probes of the present invention can specifically detect low levels of target molecules, even in the background of clinical samples. In addition, the primers should amplify the Q RNA and the Q and wt

probes show appropriate specificity for their cognate targets. Thus, the primers used in the present invention provide unexpectedly good results for the detection and quantitation of β chemokine RNA.

5 Even though the non-preferred primers of the present invention are non-preferred for a NASBA method, such primers may be useful for other amplification methods such as polymerase chain reaction (PCR) or reverse transcription/polymerase chain reaction (RT/PCR).

10 Interestingly, we have shown that the expression of mRNA encoding chemokines (e.g., RANTES, MIP-1a, MIP-1b) can be detected in patient PBMC without the need for stimulation by mitogens. Since PBMC samples examined to date for MDC indicate the need for preliminary stimulation, it follows that the expression of different chemokine genes is not regulated in the same way. Thus, assays with
15 detection and quantitation ability for chemokine RNA expression will be highly relevant to the overall assessment of patient immunocompetence.

CLAIMS

WE CLAIM:

1. An oligonucleotide of 10-50 nucleotides, comprising at least 10 consecutive nucleotides of one of the following sequences: SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, SEQ ID NO:15, SEQ ID NO:17, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:34, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:43 and SEQ ID NO:45.
2. The oligonucleotide of claim 1, which is linked to the sequence of SEQ ID NO:44 at the 5' end thereof.
3. A method for the detection of RANTES RNA in a sample, comprising:
- obtaining a sample that may contain RANTES RNA;
 - performing isothermal transcription-based amplification on the sample with two oligonucleotide primers, one of which comprises at least 10 consecutive nucleotides of a sequence selected from SEQ ID NO:2 and SEQ ID NO:4, and the other of which comprises at least 10 consecutive nucleotides of a sequence selected from SEQ ID NO:1 and SEQ ID NO:5; and
 - detecting the resulting product of step b), whereby detection of the amplification product indicates the presence of RANTES RNA in the sample.

4. The method of claim 3, wherein the amplification product is detected with a labeled wild-type probe comprising a sequence selected from SEQ ID NO:3 and SEQ ID NO:8.

5 5. The method of claim 4, further comprising adding a known amount of control RNA Q at step b), and detecting amplification product of Q by using a labeled probe comprising the sequence of SEQ ID NO:7, whereby the quantity of RANTES RNA in the sample is calculated by comparing the signals of the probe for Q and the wild-type probe.

10 6. The method of claim 3, wherein the sample comprises cells and RNA is extracted from the cells in the sample prior to step b).

15 7. A primer pair for the detection or quantitation of RANTES RNA in a sample, comprising one primer selected from SEQ ID NO:1 and SEQ ID NO:5, and one primer selected from SEQ ID NO:2 and SEQ ID NO:4.

20 8. The primer pair of claim 7, which is SEQ ID NO:2 and SEQ ID NO:5.

9. A kit for the detection or quantitation of RANTES RNA in a sample, comprising the primer pair of claim 7 and at least one probe selected from SEQ ID NO:6, SEQ ID NO:7, and SEQ ID NO:8.

25 10. A method for the detection of MIP-1 α RNA in a sample, comprising:

- 30 a) obtaining a sample that may contain MIP-1 α RNA;
b) performing isothermal transcription-based amplification on the sample with two oligonucleotide primers, one of which comprises at least 10 consecutive nucleotides of a sequence selected from SEQ ID NO:9, SEQ ID NO:10, SEQ

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ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO: 21, and SEQ ID NO:22, and the other of which comprises at least 10 consecutive nucleotides of a sequence selected from SEQ ID NO:11, SEQ ID NO:13; SEQ ID NO:14, and SEQ ID NO:23; and

- c) detecting the resulting product of step b), whereby detection of the amplification product indicates the presence of MIP-1 α RNA in the sample.

10

11. The method of claim 10, wherein the amplification product is detected with a labeled wild-type probe comprising a sequence selected from SEQ ID NO:12, SEQ ID NO:19, and SEQ ID NO:25.

15

12. The method of claim 10, further comprising adding a known amount of control RNA Q at step b), and detecting amplification product of Q by using a labeled probe comprising the sequence of SEQ ID NO:20, whereby the quantity of MIP-1 α RNA in the sample is calculated by comparing the signals of the probe for Q and the wild-type probe.

20

13. The method of claim 10, wherein the sample comprises cells and RNA is extracted from the cells in the sample prior to step b).

25

14. A primer pair for the detection or quantitation of MIP-1 α RNA in a sample, comprising one primer selected from SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:21, and SEQ ID NO:22, and one primer selected from SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:23.

30

15. The primer pair of claim 14, which is consisting of SEQ ID NO:13 and SEQ ID NO:15.

16. A kit for the detection or quantitation of MIP-1 α RNA in a sample, comprising the primer pair of claim 14 and at least one probe selected from SEQ ID NO:12, SEQ ID NO:19, SEQ ID NO:20, and SEQ ID NO:25.

5 17. A method for the detection of MIP-1 β RNA in a sample, comprising:

- a) obtaining a sample that may contain MIP-1 β RNA;
- b) performing isothermal transcription-based amplification on the sample with two oligonucleotide primers, one of which
10 comprises at least 10 consecutive nucleotides of a sequence selected from SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:41, and SEQ ID NO:42, and the other of which comprises at least 10 consecutive nucleotides of
15 a sequence selected from SEQ ID NO:28, SEQ ID NO:30; SEQ ID NO:31, SEQ ID NO:43, and SEQ ID NO:45; and
- c) detecting the resulting product of step b), whereby detection of the amplification product indicates the presence of MIP-1 β RNA in the sample.

20

18. The method of claim 17, wherein detection of the amplification product uses a labeled wild-type probe comprising a sequence selected from SEQ ID NO:29, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:39, and SEQ ID NO:40.

25

19. The method of claim 17, further comprising adding a known amount of control RNA Q at step b), and detecting amplification product of Q by using a labeled probe comprising the sequence of SEQ ID NO:38, whereby the quantity of MIP-1 β RNA in the sample is calculated
30 by comparing the signals of the probe for Q and the wild-type probe.

20. The method of claim 17, wherein the sample comprises cells and RNA is extracted from the cells in the sample prior to step b).

5 21. A primer pair for the detection or quantitation of MIP-1 β RNA in a sample, comprising one primer selected from SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:41, and SEQ ID NO:42, and one primer selected from SEQ ID NO:28, SEQ ID NO:30; SEQ ID NO:31, SEQ ID NO:43, and SEQ ID NO:45.

10

22. The primer pair of claim 21, which is SEQ ID NO:30 and SEQ ID NO:32.

15

23. A kit for the detection or quantitation of MIP-1 β RNA in a sample, comprising the primer pair of claim 21 and at least one probe selected from SEQ ID NO:29, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, and SEQ ID NO:40.

1 / 4

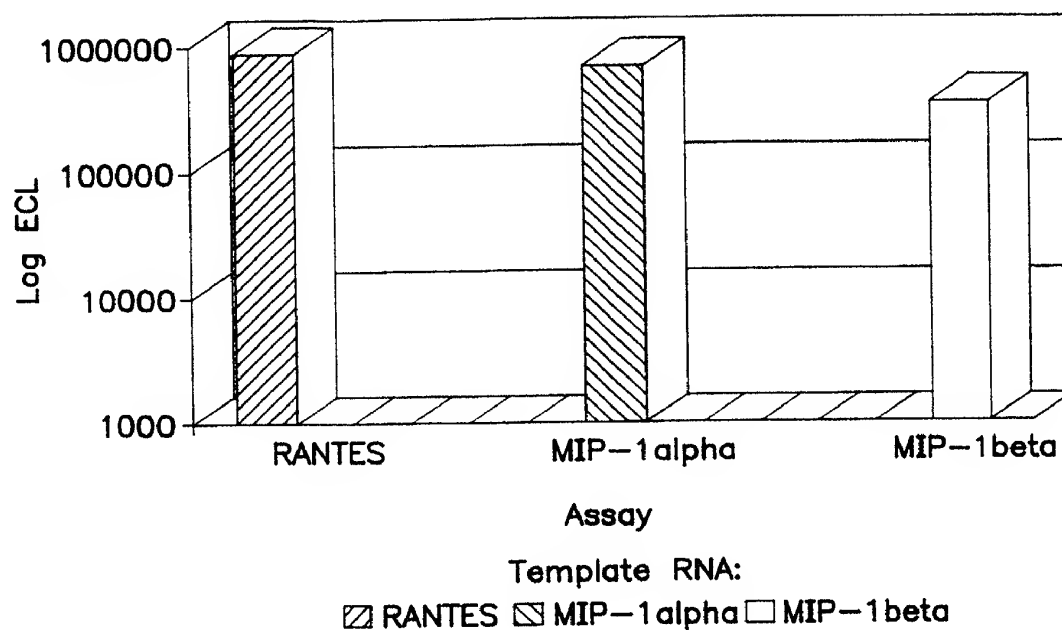


FIG. 1

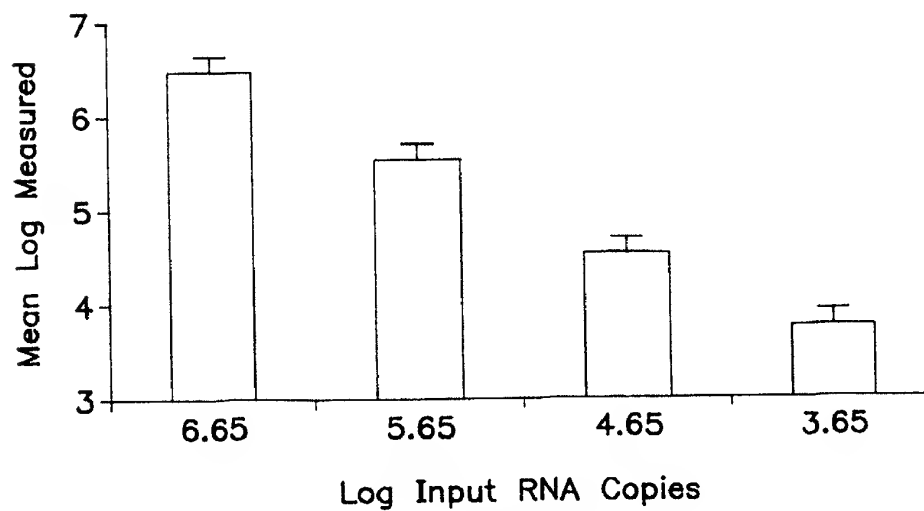


FIG. 2

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RANTES QT NASBA:
Performance in HIV-1+Patient PBMC

Patient	Mean*	SD	Patient	Mean*	SD
1	5.63	0.38	7	6.43	0.18
2	6.11	0.03	8	6.50	0.30
3	6.44	0.13	9	5.55	0.43
4	6.28	0.20	10	6.84	0.11
5	6.27	0.23	11	5.81	0.03
6	6.44	0.28	12	5.62	0.17

*Log mean value derived from individual analysis of $10^2, 10^3, 10^4$ PBMC per patient and results normalized to 10^4 PBMC.

FIG. 3

MIP-1 alpha QT NASBA:
Quantitation of In Vitro Produced RNA

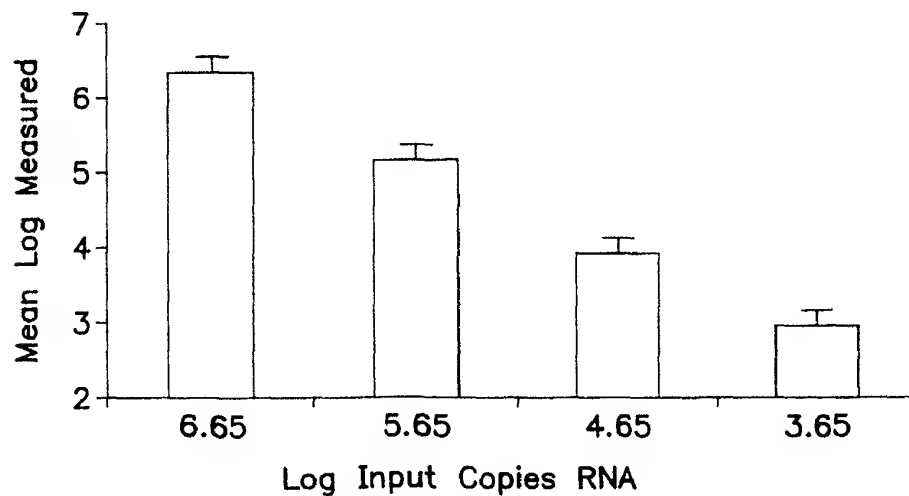


FIG. 4

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MIP-1 alpha QT NASBA:
Performance in HIV-1+Patient PBMC

Patient	Mean*	SD	Patient	Mean*	SD
1	4.76	0.00	7	6.00	0.19
2	5.10	0.06	8	5.57	0.12
3	4.80	0.06	9	5.13	0.03
4	6.16	0.09	10	5.74	0.08
5	5.85	0.06	11	5.40	0.30
6	5.87	0.10	12	6.07	0.15

*Log mean value derived from individual analysis of 10^4 , 10^5 , 10^6 PBMC per patient and results normalized to 10^6 PBMC.

FIG. 5

MIP-1 beta QT NASBA:
Performance on In Vitro Produced RNA

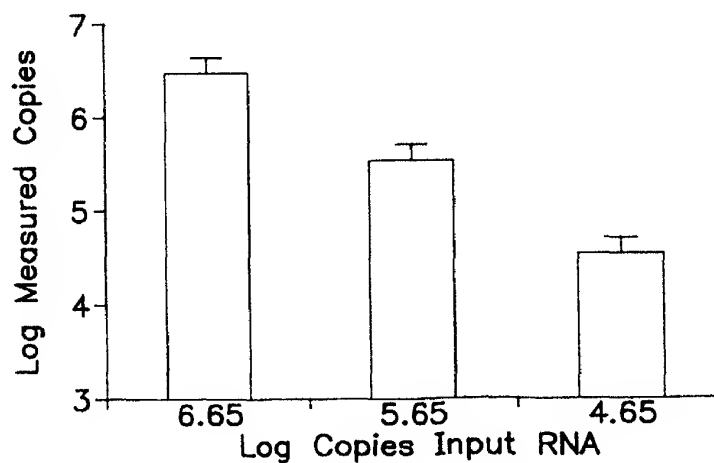


FIG. 6

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MIP-1 beta QT NASBA:
Analysis of HIV-1+Patient PBMC

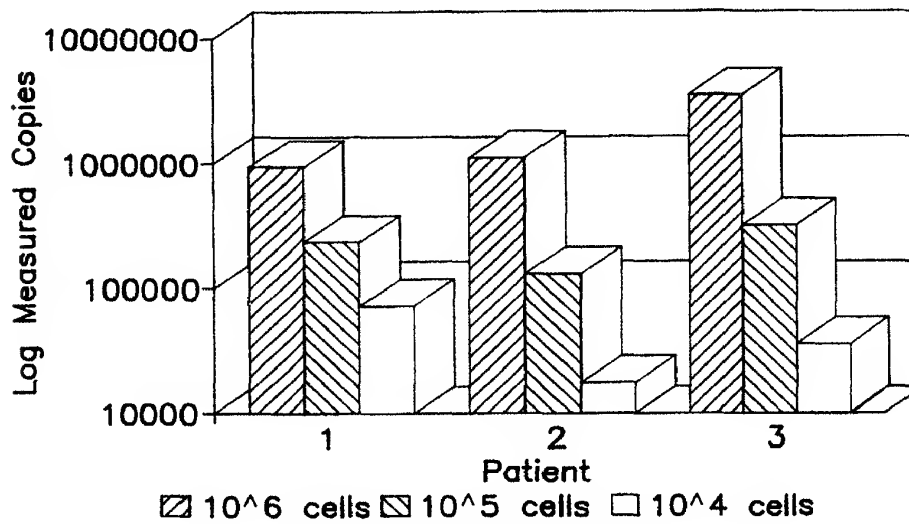


FIG. 7

RANTES and MIP-1 alpha RNA levels in
HIV-1+patients: 10⁵ PBMCs

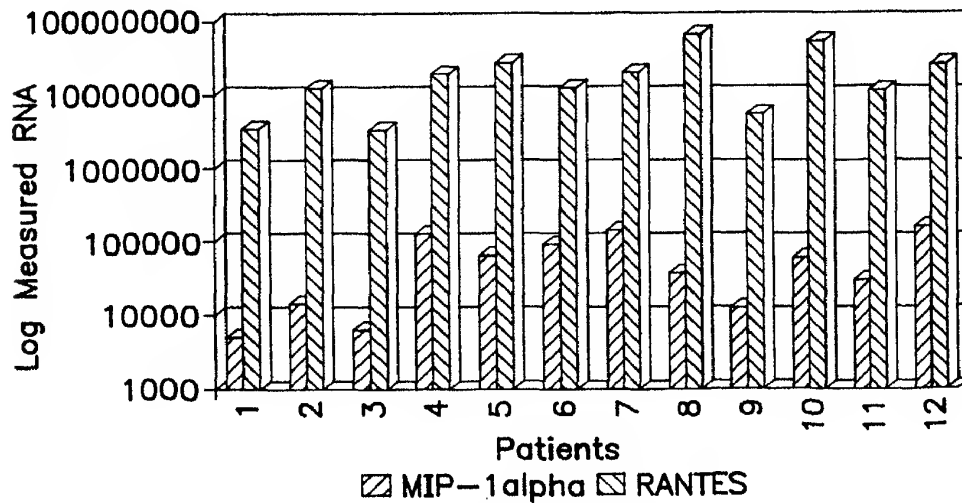


FIG. 8

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING

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WILLIAMS, KIMBERLY G.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/01327

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 668 294 A (MEAGHER RICHARD B ET AL) 16 September 1997 see table 2, SEQ ID no 21: 81% identity in 21pb overlap with Seq ID no 45 and includes a 13 consecutive bp overlap. ---	1
X	WO 94 29345 A (NOVONORDISK AS ; PEDERSEN OLUF (DK); BJOERBAEK CHRISTIAN (DK); FRED) 22 December 1994 see page 18, primer 33; a 94.1% identity in 17bp overlap with Seq ID No 43 and includes a 14 consecutive bp overlap ---	1
X	WO 93 13663 A (ABBOTT LAB) 22 July 1993 see example 17, page 19, primer 6b: 100% identity in 12 bp overlap with Seq ID No 32 --- -/--	1

☒ Further documents are listed in the continuation of box C

☒ Patent family members are listed in annex.

" Special categories of cited documents "

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

21 May 1999

Date of mailing of the international search report

04/06/1999

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Authorized officer

Osborne, H

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/01327

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
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Y	--- ROMANO J W ET AL: "NASBA A NOVEL, ISOTHERMAL DETECTION TECHNOLOGY FOR QUALITATIVE AND QUANTITATIVE HIV-1 RNA MEASUREMENTS" CLINICS IN LABORATORY MEDICINE, vol. 16, no. 1, 1 March 1996, pages 89-103, XP000600141 see the whole document	2-23
Y	--- WO 95 20681 A (INCYTE PHARMA INC) 3 August 1995 see whole document, especially table 3	2-23
Y	--- NATORI Y ET AL: "Gene expression of CC chemokines in experimental crescentic glomerulonephritis (CGN)" CLINICAL AND EXPERIMENTAL IMMUNOLOGY, vol. 109, 1997, pages 143-148, XP002103351 see summary and discussion	2-23
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Y	--- COCCHI F ET AL: "IDENTIFICATION OF RANTES, MIP-1ALPHA, AND MIP-1BETA AS THE MAJOR HIV-SUPPRESSIVE FACTORS PRODUCED BY CD8+ T CELLS" SCIENCE, vol. 270, no. 572, 15 December 1995, pages 1811-1815, XP000616644 cited in the application see abstract; Table 1 and page 1814, paragraph 2.	2-23
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In International Application No
PCT/US 99/01327

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCHMIDTMAYEROVA H ET AL: "Human immunodeficiency virus type 1 infection alters beta peptide expression in human monocytes: Implications for recruitment of leucocytes into brain and lymph nodes" PROC. NATL. ACAD. SCI. USA, vol. 93, - January 1996 pages 700-04, XP002103076 cited in the application see the whole document ---	2-23
Y	WO 97 19696 A (LUSSO PAOLO ;GALLO ROBERT C (US); COCCHI FIORENZA (US); VICO ANTHO) 5 June 1997 see page 3, line 17 - page 4, line 9 ---	2-23
Y	WO 96 23068 A (POWER CHRISTINE ANNA ;WELLS TIMOTHY NIGEL CARL (CH); GLAXO GROUP L) 1 August 1996 see page 5, paragraph 2 - paragraph 4 ---	2-23
A	BLUMAN E M ET AL: "HUMAN NATURAL KILLER CELLS PRODUCE ABUNDANT MACROPHAGE INFLAMMATORY PROTEIN-1 ASPIRE IN RESPONSE TO MONOCYTE-DERIVED CYTOKINES" JOURNAL OF CLINICAL INVESTIGATION, vol. 97, no. 12, 15 June 1996, pages 2722-2727, XP000647539 ---	
A	PATTISON J M ET AL: "RANTES chemokine expression in transplant-associated accelerated atherosclerosis" JOURNAL OF HEART AND LUNG TRANSPLANTATION, vol. 15, no. 12, 1 December 1996, pages 1194-1199, XP002094329 ---	
A	STREHLAU J ET AL: "QUANTITATIVE DETECTION OF IMMUNE ACTIVATION TRANSCRIPTS AS A DIAGNOSTIC TOOL IN KIDNEY TRANSPLANTS" PROC. NATL. ACAD. SCI. USA, vol. 94, January 1997, pages 695-700, XP002103077 cited in the application -----	

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Information on patent family members

International Application No

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